Investigating Metalloenzyme-based Signaling Pathways in Latent TB

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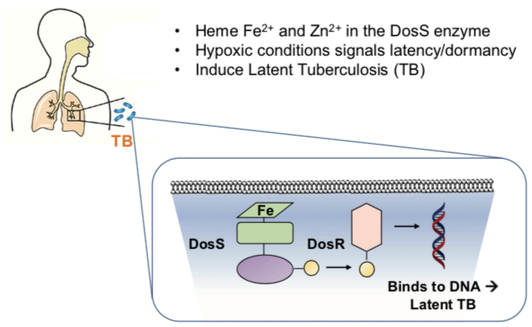
Introduction

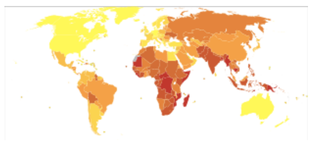
- Twenty five percent of the world's population is infected with some form of *Mycobacterium tuberculosis* (*Mtb*) causing 1.7 million Tuberculosis (TB) related deaths across the world annually.
- *Mtb* bacteria is transmitted through air and becomes infectious when inhaled into the lungs.
- The human immune system engulfs the bacteria where it encounters hypoxic conditions inside the macrophage. The *Mtb* can survive these conditions by transferring to a metabolically dormant, non-replicating latent state where anti-TB drugs do not work.
- Low levels of O_2 and high levels of NO and CO_2 turn on a metalloenzyme pathways called DosS-DosR.
- This system uses a heme iron cofactor to activate the pathway and signal the transcription of approximately 50 dormancy related genes.



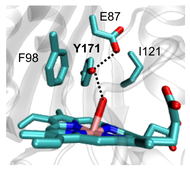
- Heme Fe^{2+} and Zn^{2+} in the DosS enzyme
- Hypoxic conditions signals latency/dormancy
- Induce Latent Tuberculosis (TB)

Aim

- The goal is to understand the structural, functional, and mechanistic basis for low oxygen affinity in the DosS heme.
- The capability of the *Mtb* to transition into a latent form is the main reason for the 6 month long treatment regimen.
- In the latent form, *Mtb* can become active again with a compromised immune system or HIV infection.
- Understanding the details of how this metalloenzyme pathway that *Mtb* employs to transition into this latent state can be used for identifying drugs and treatment that inhibits this transition.
- This information can be used to develop drugs that maintain *Mtb* in its active state and counterintuitively reduce TB treatment times and eradicate the issue of drug resistance in TB.

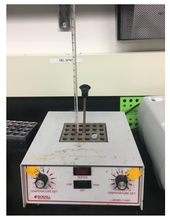


Structure of DosS heme



Expression and Purification

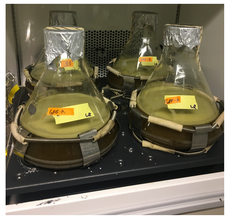
- The bacterial cells are transformed using BL21 gold-DE3 competent cells with the DosS and GroES/EL gene.
- DNA is added in equal amounts to each of the cells and the mixture is allowed 30-45 minutes to cool on ice.
- The cells are heat shocked and rescued by addition of 300μL sterile SOC media and incubated in a shaker
- The cells are plated on agar containing ampicillin and chloramphenicol and grown over night at 37℃.



Heat shocking cells for uptake of the plasmid.

Cells grown overnight after transformation.

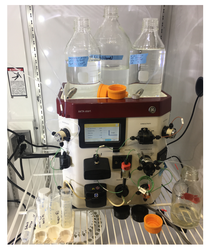
- Cultures of the cells are grown in primary and secondary cultures until the OD600 is at least 1.5.
- The cells are induced with 5-aminolevulinic acid and hemin chloride and IPTG is added.
- The cells are pelleted in the centrifuge to separate all of the supernatant from the cells.



Secondary cultures after induction.

Pelleting the culture to isolate the cells.

- Using lysis buffer and protease inhibitors, the cells are lysed and sonicated to release the cellular content.
- The cell debris is pelleted down by centrifugation and the supernatant is ran through a nickel column in the Akta start purification machine.
- The pure protein fractions are collected and dialyzed overnight.
- The protein is then concentrated down and ran through a size exclusion column (Akta pure) to further purify the protein.

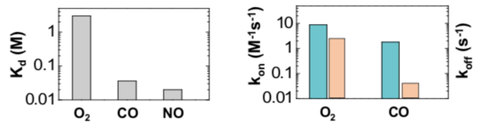


Akta start

Akta pure

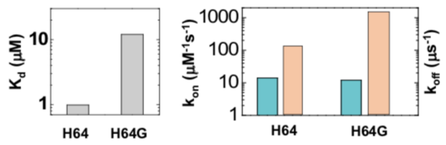
Future Directions

- The expected results after further testing the purified protein will provide information to better understand the mechanistic, functional, and structural features of the DosS heme.
- Measurements of ligand kinetics will be taken for both the wild type and the mutants.
- The affinity (K_d), association (k_{on}), and dissociation (k_{off}) will be instrumental in understanding which types of mutations influence the ligand kinetics.



Ligand recognition in DosS.

- The affinity for O_2 is much lower than CO and NO. Additionally, DosS is not able to stabilize O_2 and has a large dissociation constant.
- Introduction to mutations in the protein can alter the affinity and stabilization of O_2.

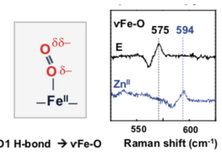


Ligand recognition in DosS with removal of His64.

- The hydrogen bonding from distal residues is an important component to oxygen stabilization.
- Mutations in close locations can alter the strength and directionality of hydrogen bonding.
- The strength of the hydrogen bonding can be determined using cryo-reduced EPR spectroscopy.
- The directionality of the hydrogen bonding can be determined using vibrational spectroscopy.

Cryo-reduced EPR Spectroscopy

- It is expected that an increased amount of hydrogen donation would lead to an increased formation of hydroperoxo.

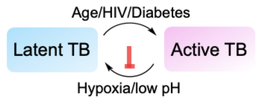


Vibrational Spectroscopy

- It is expected that an increased amount of hydrogen donation would lead to a stronger Fe-O bond.

Conclusions

- Multiple assays are needed in order to determine the structural, functional, and mechanistic basis for the low O_2 affinity in the DosS heme.
- Lots of cell cultures are required for the isolation and purification of the protein in order to run the indicated tests.
- Insights gathered from the ligand kinetics are fundamental to designing novel drugs that reduce the ability for *Mtb* to transition into the latent form.



- Being able to incorporate both the inorganic and biochemical techniques make metalloenzymes perfect targets for drug design.
- Continued work on the expression and purification of multiple versions of the DosS protein are critical to gaining a broader understanding of the molecular mechanisms.
- Systematic manipulation of hydrogen bonding from distal residues can influence the stabilization of oxygen.
- Purification of mutant proteins and different DosS domains are important to the understanding of the functional characteristics.

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